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Characterization of Malathion Resistance in a Mexican Population of *Rhizopertha dominica*

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Abstract: Malathion resistance of a field-collected population of *Rhizopertha dominica* (Coleoptera: Bostrichidae) from Mexico was evaluated and the resistance mechanisms were characterized both *in vivo* and *in vitro*. The Mexican population showed a resistance level of 50-fold at LC_{50} as compared with that of a susceptible laboratory population. Malathion bioassays with the synergists triphenyl phosphate, piperonyl butoxide and diethyl maleate suggested that esterases were likely to contribute to the resistance whereas cytochrome P450 monooxygenases and glutathione *S*-transferases were not. In-vitro assays of esterases indicated that the general esterase activity was 1·3-fold higher in the Mexican population than in the susceptible population. However, the phosphotriesterase activity in the resistant population was 3·7-fold higher than in the susceptible population. Significantly higher phosphotriesterase activity in the resistant population was further indicated by 3·4-fold increase of V_{max} in enzyme kinetics and higher frequency of individuals with high phosphotriesterase activity in this population. All these findings suggested that phosphotriesterases play a role in malathion resistance in the Mexican population of lesser grain borer.

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Key words: *Rhizopertha dominica;* malathion; insecticide resistance; phosphotriesterases; resistance mechanisms; enzyme kinetics

1 INTRODUCTION

The lesser grain borer *Rhizopertha dominica* F. (Coleoptera: Bostrichidae) is one of the most important pests of stored cereals throughout the world.[1,2] It is primarily controlled by insecticides, especially in warmer climates, due to the lack of reliable control alternatives.[1,3,4] Malathion is one of the most commonly used insecticides against stored grain insect pests,[1,4,5] and resistance to this compound in *R. dominica* has been reported in several countries.[6–9]

Although malathion resistance in *R. dominica* has been documented in Brazil and the United States,[7–9] there is a lack of information in Spanish-America where malathion resistance has been reported only in insects from Colombia.[6] No information is available about the levels and mechanisms of malathion resistance in *R. dominica* in this very large area. The objectives of this study were: (1) to determine the malathion resistance level in a field-collected population of *R. dominica* from Mexico, (2) to investigate its underlying mechanisms and (3) to compare them with our recent findings in populations of *R. dominica* from Brazil and the United States, where enhanced phosphotriesterase activity and reduced sensitivity of acetylcholinesterase were the main mechanisms of organophosphate resistance in this insect species.[10,11]

2 MATERIALS AND METHODS

2.1 Insects

Individuals of the Mexican population of *R. dominica* were collected with a pheromone trap in August 1995

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close to a storage facility of Celaya County, Guanajuato, Mexico. The standard susceptible population used in this study is maintained in the Laboratory of Stored Product Insects, Department of Entomology, Kansas State University, Manhattan, Kansas, USA. Both populations were reared on whole wheat under constant conditions (25(±2)°C and 65(±5)% RH) without any exposure to insecticides.

2.2 Chemicals

Technical-grade samples of malathion and malaoxon were provided by Cheminova Agro (Wayne, NJ, USA). The insecticide synergists triphenyl phosphate (TPP), piperonyl butoxide (PBO) and diethyl maleate (DEM) were obtained from Eastman Kodak Co. (Rochester, NY, USA), Fairfield Chemical Co. (Baltimore, MD, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively.

Technical-grade paraoxon, as well as analytical quality acetylthiocholine iodide, bicinchoninic acid (BCA) solution, 5,5′-dithio-bis(2-nitrobenzoic acid), α-naphthyl acetate, reduced glutathione, sodium dithionite, sodium dodecylsulfate and 'Triton' X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbon monoxide, 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB) were purchased from Aldrich Chemical Co. (Milwaukee, WS, USA) and bovine serum albumin and glycine were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3 Insecticide bioassays

Non-sexed adult insects were assayed by exposure to dried insecticide residues on the inner surface of glass scintillation vials (20 ml) as previously described.[3] For the synergism assays, the insects were exposed for 1 h to dried synergist residues (1 mg ml^{-1}) in glass vials prior to their exposure to insecticide residues. Four replicates of 25 insects for each concentration and six to seven insecticide concentrations were used in each bioassay; control treatments where only solvent (acetone) was applied were also included. Concentration-mortality data were subjected to probit analysis (PROC PROBIT).[12]

2.4 Preparations of insect microsomes and enzymes

Random samples of non-sexed adult insects were collected and immediately frozen at −20°C. Ten insects were homogenized in ice-cold phosphate buffer (0·1 M, pH 7·5; 1·5 ml) containing 'Triton' X-100 (3 ml $litre^{-1}$) after prior surface sterilization with potassium chloride solution (11·5 g $litre^{-1}$). The crude homogenate was centrifuged at $10\,000g_{max}$ for 15 min. The pellet was discarded and aliquots of the supernatant were taken for determination of general esterase, acetylcholinesterase (AChE) and glutathione *S*-transferase activities. For determination of phosphotriesterase (PhTE) activity, 0·05 M glycine-NaOH buffer (pH 8·0) was used for homogenizing the insects and the supernatant of $10\,000g_{max}$ centrifugation was also used as enzyme source.

Insect microsomes were prepared by homogenizing batches of insects (0·5 g) in phosphate buffer (0·1 M; 5·0 ml) with EDTA (1 mM). The supernatant from the $10\,000g_{max}$ centrifugation was further centrifuged at $105\,000g_{max}$ for 60 min in a Beckman L3-50 ultracentrifuge with posterior resuspension of the microsomal pellet in 0·1 M phosphate buffer. The resuspended microsomal fraction was used for the determination of the contents of cytochrome b_5, P450 and P450.

2.5 Determination of cytochrome b_5, P420 and P450 contents and enzyme activities

The contents of cytochrome b_5, P420 and P450 were determined following Omura and Sato.[13] General esterase activity was determined using a microplate format and α-naphthyl acetate as substrate.[14] AChE activity and inhibition by malaoxon were assayed by Ellman's method[15] with some modifications.[10] Glutathione *S*-transferase activity was assayed by measuring the conjugation of two different substrates, CDNB and DCNB,[16,17] since the activity towards these may vary among different isozymes of glutathione *S*-transferases.[18,19]

PhTE activity was determined using paraoxon as substrate to detect the released *p*-nitrophenol colorimetrically at 405 nm, based on the method of Guedes *et al.*[11] with slight modifications. Briefly, 50 μl of insect homogenate was placed in a well of a 96-well flat-bottom microtitre plate containing 100 μl paraoxon in buffer at a final concentration of 1 mM. Activity was measured at 405 nm before and after 1 h incubation at 37°C in a Vmax kinetic microplate reader utilizing SOFT-max software (Molecular Devices Corp., Menlo Park, CA, USA). Protein concentration was determined by the BCA method using bovine serum albumin as standard.[20] All data obtained were subjected to Student's *t*-test ($P < 0{\cdot}05$) or regression analysis (PROC REG)[12] whenever appropriate.

3 RESULTS

3.1 Insecticide bioassays

Concentration–mortality regression lines obtained by probit analysis are presented in Table 1. The malathion resistance ratio for the Mexican population of *R. dominica* was 50-fold at the LC_{50}. TPP was the only synergist able to significantly potentiate the toxicity of malathion based on the criterion of failure of 95% CL to overlap.

TABLE 1
Effect of the Synergists Triphenyl Phosphate (TPP), Piperonyl Butoxide (PBO) and Diethyl Maleate (DEM) on the Toxicity of Malathion to Susceptible and Mexican Populations of *Rhizopertha dominica*

Insecticide	*Population*	*Slope* (±*SEM*)	LC_{50} (95% *CL*) ($\mu g\ cm^{-2}$)	LC_{50} *RR*[a]	LC_{50} *SR*[b]	χ^2	*Probability*
Malathion	Susceptible	0·32 (±0·04)	0·031 (0·023–0·043)	—	—	5·9	0·20
	Mexican	0·40 (±0·03)	1·513 (1·012–2·270)	48·8	—	14·0	0·06
Malathion + TPP	Susceptible	0·24 (±0·02)	0·003 (0·002–0·005)	—	10·3	3·1	0·67
	Mexican	0·55 (±0·03)	0·081 (0·071–0·093)	27·0	18·7	6·0	0·30
Malathion + PBO	Susceptible	0·45 (±0·03)	0·066 (0·043–0·100)	—	0·47	8·3	0·08
	Mexican	0·34 (±0·03)	3·771 (2·503–5·921)	57·1	0·40	9·1	0·11
Malathion + DEM	Susceptible	0·52 (±0·03)	0·031 (0·024–0·041)	—	1·0	11·0	0·06
	Mexican	0·33 (±0·03)	2·775 (2·185–3·655)	89·5	0·55	2·5	0·64

[a] Resistance ratio (= LC_{50} Mexican population ÷ LC_{50} susceptible population).
[b] Synergism ratio (= LC_{50} unsynergized malathion ÷ LC_{50} synergized malathion).

In contrast, DEM did not show significant synergism to malathion and PBO was rather an antagonist, significantly decreasing the toxicity of malathion, especially for the Mexican population, based on the same criterion of failure of 95% CL to overlap. No synergist was able to suppress completely the resistance to malathion.

3.2 Enzyme activities and cytochrome b_5, P420 and P450 contents

Specific activities of enzymes potentially associated with malathion resistance were determined in both Mexican and susceptible populations of *R. dominica* (Table 2). AChE and glutathione *S*-transferase activities were similar in both populations. However, specific activity of general esterases was 1·3-fold higher in the Mexican population of *R. dominica*, in agreement with our synergism data, showing that the malathion toxicity could be synergized at a greater level in the Mexican population than in the susceptible population. PhTE activity in the Mexican population was 3·7-fold higher than in the susceptible population, suggesting that increased PhTE activity in the Mexican population plays a major role in conferring malathion resistance.

The levels of cytochrome P450 and b_5 were 1·3- and 1·6-fold higher, respectively, in the Mexican than in the susceptible population, whereas the level of cytochrome P420 was significantly higher in the susceptible population. Cytochrome P420 is a breakdown product of cytochrome P450, suggesting that the susceptible population may actually have a higher cytochrome P450 content than the Mexican population. The higher level of cytochrome P450 in the susceptible population may have increased its instability in the relatively crude preparations used in this study. The lower cytochrome

TABLE 2
Comparisons of Hydrolases and Glutathione *S*-Transferase Activities, and Cytochrome b_5, P450 and P420 Contents (± SEM) between the Susceptible and Mexican Populations of *Rhizopertha dominica*[a]

Enzyme	*Populations*		*M*/S
	Susceptible	*Mexican*	
Hydrolases			
General esterases (nmol min^{-1} mg^{-1} protein)	18·59 (±0·44)	25·07 (±1·19)*	1·3
Phosphotriesterases (nmol min^{-1} mg^{-1} protein)	0·10 (±0·02)	0·37 (±0·07)*	3·7
Acetylcholinesterase (nmol min^{-1} mg^{-1} protein)	27·92 (±5·40)	24·25 (±3·57)	0·9
Glutathione *S*-transferases			
CDNB conjugation (nmol min^{-1} mg^{-1} protein)	84·13 (±10·19)	102·72 (±13·63)	1·2
DCNB conjugation (nmol min^{-1} mg^{-1} protein)	4·84 (±0·96)	8·08 (±1·18)	1·7
Cytochrome b_5, P450 and P420 contents			
Cytochrome P450 (pmol mg^{-1} protein)	43·16 (±3·32)	57·39 (±1·97)*	1·3
Cytochrome P420 (pmol mg^{-1} protein)	76·91 (±10·99)	19·65 (±4·91)*	0·3
Cytochrome b_5 (pmol mg^{-1} protein)	115·95 (±7·12)	184·97 (±5·42)*	1·6

[a] * = Means are significantly different from those of the susceptible population by Student's *t*-test ($P < 0·05$; $n = 3$).

TABLE 3
Comparisons of Kinetic Parameters (± SEM) of General Esterases, Acetylcholinesterase and Phosphotriesterases between Susceptible and Mexican Populations of *Rhizopertha dominica*[a]

Enzyme	*Population*	K_m (μM)	V_{max} (*nmol* min^{-1} mg^{-1})
General esterases	Susceptible	4·88 (±0·33)	23·19 (±0·30)
	Mexican	5·61 (±1·05)	27·00 (±2·79)
Acetylcholinesterase	Susceptible	5·10 (±0·86)	28·25 (±2·54)
	Mexican	6·11 (±0·29)	27·01 (±3·43)
Phosphotriesterases	Susceptible	(3·74 (±0·79)) × 10^3	0·50 (±0·08)
	Mexican	(3·48 (±0·51)) × 10^3	1·68 (±0·37)*

[a] * Means are significantly different by Student's *t*-test ($P < 0{\cdot}05$; $n = 3$).

P450 content in the resistant population is another potential resistance mechanism, because this population would convert less of the dose of malathion to malaoxon (the active toxicant) than the susceptible population. However, the similar levels of antagonism observed in both populations in in-vivo bioassays with PBO does not provide support for this hypothesis.

Table 3 presents the estimated kinetic parameters, K_m and V_{max}, of general esterases, AChE and PhTE for the susceptible and Mexican populations of *R. dominica*. There was no difference in kinetic parameters of general esterases and AChE between the two populations, but PhTEs from the Mexican population showed 3·4-fold higher hydrolysing efficiency towards paraoxon, as measured by the V_{max} values, than the susceptible population (Fig. 1). Furthermore, the Mexican population showed higher heterogeneity of distribution and higher frequency of individuals with high PhTE activity than did the susceptible population (Fig. 2), which would be expected in a field-collected population presenting enhanced PhTE activity as a major insecticide-resistance mechanism.

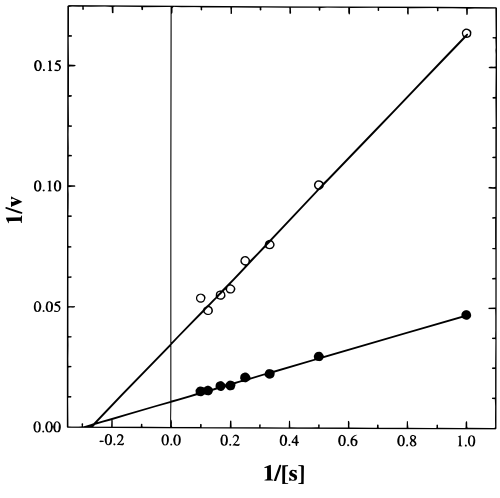

Fig. 1. Lineweaver–Burk (double reciprocal) plot of $1/v \times 1/[s]$ for phosphotriesterases from (○) susceptible and (●) Mexican populations of *Rhizopertha dominica*. Each point is based on the mean of three replicates. Coefficients of determination (R^2) > 0·70 ($P < 0{\cdot}001$).

Inhibition of AChE by malaoxon in susceptible and Mexican *R. dominica* was similar (Fig. 3). I_{50} values were also similar in both populations (2·73 (±1·42) μM for the susceptible and 4·06 (±0·90) μM for the Mexican *R. dominica*). There was no significant difference of sensitivity of AChE to inhibition by malaoxon between the two populations, which eliminates the possibility of the involvement of target-site insensitivity contributing to malathion resistance in the Mexican population of *R. dominica*.

4 DISCUSSION

Malathion resistance was characterized in a Mexican population of *R. dominica* by both in-vivo and in-vitro assays. Results from the malathion bioassays with synergists suggest that only esterases play a role in detoxifi-

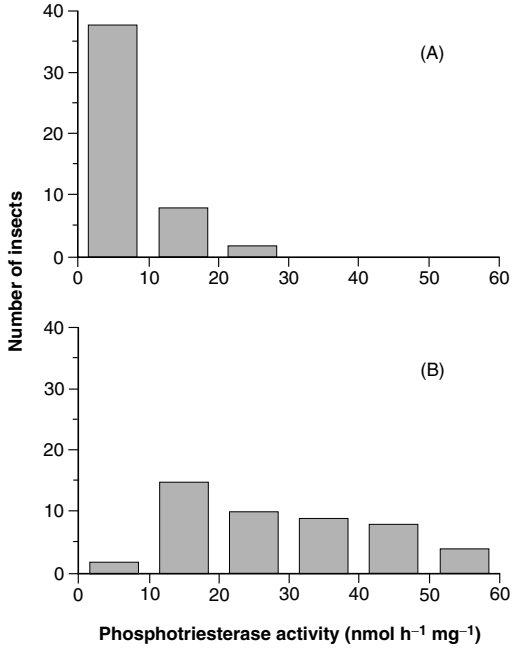

Fig. 2. Frequency of distribution of phosphotriesterase activity in (A) susceptible and (B) Mexican populations of *Rhizopertha dominica*. A total number of 48 insects were individually assayed in each population.

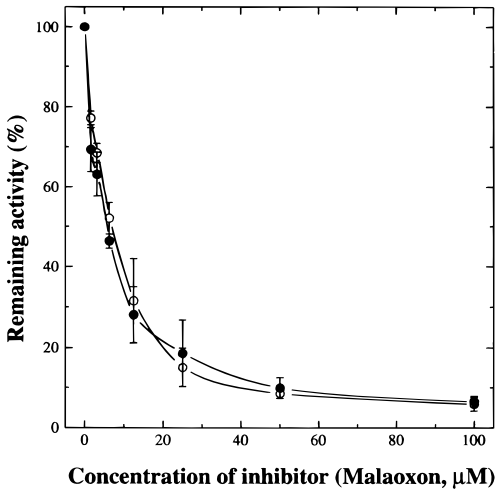

Fig. 3. Remaining activity (%) of acetylcholinesterase when subjected to inhibition by malaoxon in (○) susceptible and (●) Mexican populations of *Rhizopertha dominica*. Each point is based on the mean of three replicates. Vertical bars indicate standard errors of the mean.

cation of malathion in both susceptible and resistant populations, since the toxicity of malathion was synergized by TPP (an esterase inhibitor) in both populations. However, relatively higher synergism of malathion by TPP in the resistant population, as compared with that in the susceptible, suggested that esterases might play a role in conferring malathion resistance in the Mexican population. In contrast, PBO showed an antagonistic effect on malathion toxicity in both populations. Because malathion requires activation by the cytochrome P450 system to yield malaoxon in order to inhibit AChE, the antagonistic effect of PBO in both the Mexican and susceptible populations apparently was due to the inhibition of cytochrome P450 monooxygenase system by PBO, resulting in the decrease of activation of malathion to malaoxon. However, it appeared clear that neither cytochrome P450 monooxygenases nor glutathione *S*-transferases contributed to malathion resistance in the Mexican population, which was demonstrated by in-vivo and in-vitro assays.

The partial suppression of malathion resistance by TPP in the Mexican *R. dominica* coupled with a small increase (*c*.30%) in general esterase activity and no significant change in the kinetic parameters of general esterases further support our hypothesis that esterases, probably mainly carboxylesterases, play only a secondary role in conferring malathion resistance. In contrast, the 3·7-fold increase of PhTE activity associated with a similar increase of the hydrolysing efficiency of this enzyme and the higher frequency of individuals with high PhTE activity in the resistant population suggested a major involvement of PhTE in malathion resistance in *R. dominica*. The higher catalytic activity of PhTEs as judged by the V_{max} values towards paraoxon, with no change in the affinity as judged by the K_m values, suggests that quantitative rather than qualitative changes in PhTEs contribute to malathion resistance in the Mexican population of *R. dominica*. The increased PhTE activity conferring organophosphate resistance has also been demonstrated in the tobacco budworm (*Heliothis virescens* F.) and the lesser grain borer (*R. dominica*) populations from Brazil and the United States.[11,21]

Previous investigations on malathion resistance in *R. dominica* relied primarily upon the use of discriminating concentrations and the use of the insecticide synergist TPP.[6–11] Such studies led to a general belief that detoxification by carboxylesterases is the main resistance mechanism in *R. dominica*.[6–10] However, our previous studies on all possible biochemical mechanisms of organophosphate resistance in Brazilian and US populations of *R. dominica* did not support such a contention.[10,11] Furthermore, results from this study on malathion resistance in a Mexican population of *R. dominica* also did not provide much evidence of the increased carboxylesterase activity as a major resistance mechanism.

Our previous studies showed that enhanced PhTE activity and reduced sensitivity of AChE to inhibition by organophosphates were major resistance mechanisms in Brazilian and US populations of *R. dominica* resistant to organophosphates.[10,11] All these, together with this finding, appear to suggest that enhanced activity of PhTEs is a common organophosphate-resistance mechanism in *R. dominica*. Since we used paraoxon, an organophosphate insecticide, directly as substrate for PhTEs, it is logical to expect that higher hydrolysing activity of PhTE towards paraoxon may reflect their hydrolysing ability to other organophosphates, especially those with similar structure. In addition, the presence of this mechanism in all organophosphate-resistant populations of *R. dominica* so far investigated, in three different American countries (i.e. Brazil, Mexico and the United States), suggests that it might be widespread in populations of *R. dominica* throughout the Americas.

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